MICROBIAL GENOMICS

RESEARCH ARTICLE

Ghazali *et al.*, *Microbial Genomics* 2021;7:000527

DOI 10.1099/mgen.0.000527

Whole-genome comparative analysis of Malaysian *Burkholderia pseudomallei* clinical isolates

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Abstract

Burkholderia pseudomallei, a soil-dwelling Gram-negative bacterium, is the causative agent of the endemic tropical disease melioidosis. Clinical manifestations of *B. pseudomallei* infection range from acute or chronic localized infection in a single organ to fulminant septicaemia in multiple organs. The diverse clinical manifestations are attributed to various factors, including the genome plasticity across *B. pseudomallei* strains. We previously characterized *B. pseudomallei* strains isolated in Malaysia and noted different levels of virulence in model hosts. We hypothesized that the difference in virulence might be a result of variance at the genome level. In this study, we sequenced and assembled four Malaysian clinical *B. pseudomallei* isolates, UKMR15, UKMPMC2000, UKMD286 and UKMH10. Phylogenomic analysis showed that Malaysian subclades emerged from the Asian subclade, suggesting that the Malaysian strains originated from the Asian region. Interestingly, the low-virulence strain, UKMH10, was the most distantly related compared to the other Malaysian isolates. Genomic island (GI) prediction analysis identified a new island of 23 kb, GI9c, which is present in *B. pseudomallei* and *Burkholderia mallei*, but not *Burkholderia thailandensis*. Genes encoding known *B. pseudomallei* virulence factors were present across all four genomes, but comparative analysis of the total gene content across the Malaysian strains identified 104 genes that are absent in UKMH10. We propose that these genes may encode novel virulence factors, which may explain the reduced virulence of this strain. Further investigation on the identity and role of these 104 proteins may aid in understanding *B. pseudomallei* pathogenicity to guide the design of new therapeutics for treating melioidosis.

DATA SUMMARY

(1) The genome assembly of *Burkholderia pseudomallei* UKMR15 has been deposited in the European Nucleotide Archive: accession number ERS3410623.

(2) The genome assembly of *B. pseudomallei* UKMPMC2000 has been deposited in the European Nucleotide Archive: accession number ERS3410624.

(3) The genome assembly of *B. pseudomallei* UKMD286 has been deposited in the European Nucleotide Archive: accession number ERS3410625.

(4) The genome assembly of *B. pseudomallei* UKMH10 has been deposited in the European Nucleotide Archive: accession number ERS3410626.

(5) The other *B. pseudomallei* genome sequences utilized for the genome comparative analysis are listed in Table S1 (available with the online version of this article). All sequences were downloaded from the National Center for Biotechnology Information (NCBI) genome database.

INTRODUCTION

Burkholderia pseudomallei is the causative agent of the tropical disease melioidosis. The prevalence of melioidosis is most frequently reported in Northern Australia and South-East Asia, typically Thailand, Malaysia and Singapore. The

Received 21 September 2020; Accepted 13 January 2021; Published 10 February 2021

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Keywords: *Burkholderia pseudomallei*; comparative genomics; genomic island; virulence factors.

Abbreviations: CDS, coding sequence; GI, genomic island; HGT, horizontal gene transfer; LCBs, locally collinear blocks; MLST, multilocus sequence type; ST, sequence type; TA, toxin–antitoxin.

The European Nucleotide Archive study accession number for the whole-genome sequences of the four Malaysian *B. pseudomallei* strains is PRJEB3252. The genome sequence accession numbers are ERS3410623 (UKMR15), ERS3410624 (UKMPMC2000), ERS3410625 (UKMD286) and ERS3410626 (UKMH10).

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Eight supplementary tables are available with the online version of this article.

bacterium can survive in diverse environments including soil and water [1, 2], and is capable of infecting both animals and humans. Reported clinical manifestations observed with human melioidosis range from an acute or chronic localized infection involving a single organ to fulminant septicaemia in multiple organs. Infection occurs when bacteria-contaminated soil comes into contact with punctured skin, or through inhalation of bacteria-contaminated dust or whilst swimming in water bodies that harbour the bacteria. The diverse clinical manifestations of melioidosis have made diagnosis difficult for untrained laboratory personnel. Treating the disease is also a challenge, as the bacteria is intrinsically resistant to a large number of antibiotics, while recurrence and relapse are common with melioidosis patients [3].

The broad range of clinical presentations observed with melioidosis patients is attributed to host risk factors and the route of infection [4, 5]. In addition, varied clinical symptoms observed with melioidosis patients may also be related to different levels of virulence exhibited by *B. pseudomallei* isolates, which, in turn, is related to genome-level differences between *B. pseudomallei* strains [6]. The first sequenced *B. pseudomallei* genome was strain K96243, which established that the bacterial genome is complex and large, consisting of two chromosomes of 4.07 and 3.17 Mbp [7]. *B. pseudomallei* strains show evidence of horizontal gene transfer (HGT) events within the genome [8], demonstrated by the presence of genomic islands (GIs). GIs shape the diversity within *B. pseudomallei* genomes leading to unique phenotypes, and affect bacterial fitness in response to the environmental niche [9]. The bacterial genome has been suggested to be an open genome, whereby sequencing of more *B. pseudomallei* strains may lead to the discovery of new genes. As noted above, genomic differences among strains most likely account for different levels of virulence among *B. pseudomallei* strains [10]. Therefore, the availability of more complete genomes of *B. pseudomallei* strains will be useful to predict new virulence factors for this organism, which would be beneficial for the design of effective treatments for melioidosis.

In a previous study, we noted that *B. pseudomallei* strains isolated from melioidosis patients in Malaysia exhibited differing virulence capacity in infection models [11, 12]. Three isolates, UKMR15, UKMPMC2000 and UKMD286, killed mice and nematode infection models at almost similar rates [mean time-to-death (TD_{mean}) of 2 days (mice)/27–31 h (nematode)], while the fourth isolate, UKMH10, was much less virulent in both models [TD_{mean} of >10 days (mice)/36 h (nematode)]. In this study, we addressed the possibility that the differing virulence was attributable to alterations at the genome level of these isolates. Hence, all four genomic DNA samples were sequenced, assembled and subjected to comparative genome analysis between these strains as well as with other fully sequenced *B. pseudomallei* genomes. Additionally, comparative analysis between the four local isolate genomes identified differences in gene content that may explain the varying levels of virulence.

Impact Statement

Burkholderia pseudomallei, a soil-dwelling bacterium, is the causative agent of melioidosis, a fatal infectious disease of humans and animals. Currently, there is no vaccine for melioidosis, and treatment is reliant on combinatorial antibiotic therapy. The bacterium has a large genome consisting of two chromosomes carrying genes that encode proteins with important roles in survival in diverse environments, as well as in the infected host. Furthermore, different *B. pseudomallei* isolates may exhibit different levels of virulence. To address this, we undertook comparative genome analysis of four Malaysian clinical isolates with different virulence capacity. Phylogenomic analysis of the assembled genomes with *B. pseudomallei* genomes globally showed that Malaysian strains were closely related to the Asian clade. The genome of UKMH10, the least virulent strain, was missing 104 genes compared to the other three strains, which are more virulent in different animal models. These genes may encode *B. pseudomallei* virulence factors that were previously unidentified or proteins required for adaptation in different environments. These proteins are a potential resource for understanding the complete pathogenic and/or adaptation mechanism of this pathogen. The proteins may also be ideal targets for designing antimicrobials against bacterial pathogens.

METHODS

DNA extraction, sequencing and assembly

Genomic DNA of the four *B. pseudomallei* strains, UKMD286, UKMH10, UKMPMC2000 and UKMR15, was extracted using a MasterpureDNA purification kit (Epicentre). The purity of the extracted DNA was assessed with a NanoDrop spectrophotometer (Thermo Scientific). The extracted DNA samples were submitted to a commercial partner to prepare 20 kb insert libraries for sequencing on the PacBio RS II platform. The sequence reads were assembled using PacBio Hierarchical Genome Assembly Process 2.0 (HGAP 2.0) [13] available at the SMRT Portal v2 [13]. The assembly was improved and errors were corrected by Quiver within the SMRT Portal. The completeness of the polished assemblies was assessed using BUSCO v4.1.1 [14], utilizing burkholderiales_odb10 (*N*=688) BUSCO profiles.

Multilocus sequence type (MLST) analysis

MLST assignment of the four *B. pseudomallei* isolates was undertaken using the assembled whole-genome sequences submitted to the Bacterial Isolates Genome Sequence database (BIGSdb) tool accessible on the *B. pseudomallei* MLST website (http://pubmlst.org/bpseudomallei/) [15]. Allele profiles were analysed for each strain and the sequence type (ST) was then assigned.

Genome annotation and comparative analysis

Gene prediction on all assembled genomes was performed using the *ab initio* gene predictor components of the CG-pipeline [16] using default parameters. A cut-off of 33 amino acids was applied in predicting coding sequences (CDSs). To increase the accuracy and to minimize false negatives during gene prediction, annotations derived from the *B. pseudomallei* K96243 strain reference genome were transferred onto the newly assembled genomes using RATT [17], while tRNAs and rRNAs were identified using tRNAscan-SE [18] and RNAmmer [19], respectively. Predicted genes were annotated through a Basic Local Alignment Search Tools (BLAST) search against the National Center for Biotechnology Information (NCBI) non-redundant (NR) and Swiss-Prot (SP) protein databases at a cut-off *E* value of $1\times^{-5}$, and also against the Gene Ontology (GO) [20] and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [21]. For structural comparison, complete genomes were aligned using progressiveMauve [22].

Identification of homologous clusters

The identification of orthologous groups is an essential process for comparative genomics and taxonomic analysis between sub-species genomes. The predicted protein-encoding sequences of all four strains and the reference strain K92643 were subjected to homology searches using OrthoMCL [23] with default parameters. The program groups all protein-encoding sequences into homologous clusters based on amino acid sequence similarity. Reciprocal BLAST was carried out to define putative orthologues and paralogues (between and within strains). Data from the BLAST searches were analysed using the Markov cluster algorithm (MCL) to construct homologous clusters. The final output of OrthoMCL was a gene homologue matrix describing the genic relationships between strains, including core orthologous clusters represented by orthologous genes present in all strains. Genes without corresponding orthologues from other strains were regarded as strain-specific. The number of represented gene members within an orthologue cluster may vary between strains due to gene duplication events and the existence of paralogues.

Phylogenomic analysis

Phylogenetic diversity analysis of the *B. pseudomallei* strains was conducted using publicly available genomes of clinical *B. pseudomallei* isolates and the four genomes of the Malaysian *B. pseudomallei* strains generated in this study. Core-genome SNPs were identified using Panseq [24, 25], with the core genome threshold set at 100%. Phylogenetic analysis based on the 281051 core genome SNPs was performed using MrBayes v3.2.2 [26, 27]. The analysis was conducted by sampling across the entire general time reversible (GTR) model space with gamma-distributed rate variation across sites and a proportion of invariable sites [28]. Input alignments were carried out with a sampling frequency of 500 generations. A burn-in of 25% from the beginning of the cold chain was discarded. The mean standard deviation of split frequencies was 0.027. A generation versus log probability plot of the data did not show any noticeable trend, and a potential scale reduction factor (PSRF) close to 1.0 was set for all parameters. The tree was evaluated by Bayesian posterior with a probability run of one million generations. The phylogram was rooted using *Burkholderia thailandensis* E264, and visualized and annotated using Interactive Tree of Life (iTOL) (https://itol.embl.de/) [29].

GI prediction and comparative genomics

GIs were predicted using the web computational tool Island-Viewer 4, which uses two different GI prediction methods: SIGI-HMM, which uses a hidden Markov model (HMM) and measures codon usage to identify possible GIs; and IslandPath-DIMOB, which visualizes dinucleotide sequence composition bias and the presence of mobility genes (http://www.pathogenomics.sfu.ca/islandviewer/) [30]. To identify sequence similarity of genes in GIs of the four isolates, gene sequences that reside in the GIs were retrieved from each genome and subjected to a BLASTN search against the gene and genome sequences from other isolates. Circos v0.67 [31] was used to visualize the sequence similarity between the isolates.

RESULTS

Sequencing details and assembly

The four Malaysian *B. pseudomallei* strains, UKMR15, UKMPMC2000, UKMD286 and UKMH10, were sequenced on the PacBio RS II platform using a 20 kb insert library with 200× sequence coverage. Genome assembly of the Malaysian strains successfully generated two large contigs representing chromosomes 1 and 2 for UKMR15, UKMPMC2000 and UKMD286. The UKMH10 assembled genome contained a single gap of approximately 70 kb within chromosome 2. The total genome size for all strains ranged between 7102313 and 7287440 bp with a G+C content of 68.09–68.2 mol%. Based on burkholderiales_odb10 BUSCO profiles, the completeness of the genome assemblies achieved 99.1–99.7%. As expected, no plasmids were present in any of the isolates [32]. The total gene content of the assembled genomes ranged from 5997 to 6314 CDSs, which is similar to the K96243 *B. pseudomallei* reference strain (accession numbers CP009538.1 and CP009537.1) [7]. A summary of the assembly and annotations is shown in Tables 1 and S2.

Whole-genome MLST analysis of the Malaysian strains identified three STs: ST289 (UKMR15 and UKMD286), ST84 (UKMPMC2000) and ST46 (UKMH10). All the identified STs have been previously reported for other Malaysian *B. pseudomallei* clinical isolates. When the allele sequences were compared, it was apparent that ST84 is a single-locus variant of ST289, suggesting that UKMR15, UKMD286 and UKMPMC2000 are closely related. However, UKMH10 with the ST46 allele profile is distantly related to the other three strains. Using the B. *pseudomallei* PubMLST database as a resource, we attempted to identify the likely origin of the STs identified in this study. All ST289 and ST84 strains deposited

Table 1. Genome assembly statistics and ST identification of Malaysian *B. pseudomallei* strains

The genome for three Malaysia *B. pseudomallei* strains was successfully assembled into two complete contigs representing both chromosomes, while the UKMH10 strain contained a single gap.

B. pseudomallei strain	Assembly size (bp)	No. of chromosomes	ST	CDS	G+C (mol%)	Assembly status	Accession no.
UKMR15	7179387	2	289	6172	68.20	Complete	ERS3410623
UKMPMC2000	7187124	2	84	6091	68.15	Complete	ERS3410624
UKMD286	7102313	2	289	5997	68.19	Complete	ERS3410625
UKMH10	7287440	2	46	6314	68.09	1 gap	ERS3410626

in the database are either clinical or environmental isolates originally from Southern Thailand (Songkhla, Hat Yai dan Patalung), Peninsular Malaysia and Singapore. The only exception was two isolates of ST84 identified from Australian patients with unknown travel history [33]. The majority of *B. pseudomallei* strains designated as ST46, however, are more widely dispersed throughout the Asian region, including Malaysia, China, Vietnam, Thailand and Cambodia.

Variation in chromosomal organization

Multiple genome comparison approaches were used to assess for potential chromosomal rearrangement within the Malaysian strains to address our hypothesis that potential chromosomal level differences could account for the variation in the virulence of UKMH10 compared to the other Malaysian strains. We used the genomes of five *B. pseudomallei* clinical isolates from various locations around the world, in addition to the *B. pseudomallei* K96243 genome (chromosome 1 CP009538, chromosome 2 CP009537) as the reference genome. Full details of the *B. pseudomallei* strains used in the analysis are available in Table S3. A preliminary analysis using the *B. pseudomallei* K96243 genome sequence generated by Holden *et al.* [7] identified a large, inverted region on chromosome 1 of the original K96243 genome, but not in other *B. pseudomallei* strains (data not shown). To verify whether the inverted region was a misassembly arising from limitations of the Sanger sequencing platform and ARTEMIS assembly and annotation software, the K96243 genome sequenced by Los Alamos National Laboratory (USA) was included in the comparison [32]. The comparative analysis clarified that the inverted region is only unique to the Sanger-derived K96243 sequence from position 1617487 to 2506637 bp.

Mauve is routinely used to determine global genome rearrangements that may have been a result of genome evolution. Mauve generates locally collinear blocks (LCBs) within compared genomes, which are conserved segments that are internally free from significant rearrangements. Genome alignment showed both chromosome 1 and 2 were highly syntenic across all *B. pseudomallei* strains as depicted by large LCBs. Variations in chromosomal organization were largely represented by small LCBs (<10 kb) and non-aligned regions (white spaces) across the alignment, indicating the presence of strain-specific sequences (Fig. 1). This strain-specific variation may help to shape bacteria fitness and survival. UKMH10 appeared to contain more and larger non-aligned white spaces indicating strain-specific regions that might have been acquired from other micro-organisms. It is possible that UKMH10 was more susceptible to higher HGT events compared to the other strains in this study.

Phylogenomic analysis

Phylogenomic analysis of *B. pseudomallei* focusing on human clinical isolates from various geographical regions was conducted to study the relationship of *B. pseudomallei* from Malaysia to strains from other regions. Phylogenomic analysis was based on assembled genomes available in public databases. In total, 14 genomes of *B. pseudomallei* from Malaysia together with 86 *B. pseudomallei* genomes from other regions were used in the analysis. All genomes used in the analysis are listed in Table S1. The reconstructed tree showed clear divergence according to region (Australian, South American, Micronesian and Asian clades), with every clade supported by a Bayesian posterior probability value of 75 and above (Fig. 2). All strains were clustered according to geographical origin except for the Australia strain MSHR5858, which was hypothesized to be a recent introduction into Australia from Asia [34]. Within the Asian clade, no distinct cluster representing the country of origin was observed, with strains sharing nodes and clustered with isolates from other Asian countries. All Malaysian strains were clustered within the Asian clade. For the Malaysian *B. pseudomallei* strains in this study, UKMR15, UKMPMC2000 and UKMD286 are clustered within one clade but separated from UKMH10, which may account for the difference in virulence.

GI features among Malaysian strains compared to K96243

The presence of accessory genes on mobile elements and GIs may also contribute to phenotypic and clinical differences such as virulence [35, 36]. GI prediction of the Malaysian strains was done using the IslandViewer 4 tools, IslandPath-DIMOB and SIGI-HMM [30]. GI prediction accuracy and consistency are crucial; hence, prior to predicting the GIs present in the Malaysian strains, we tested the accuracy of IslandViewer GI prediction on the Sanger K96243 genome and compared the results against the previously identified K96243 GIs [7, 9]. From our test, IslandViewer predicted the presence of 24 GIs on the K96243 genome, where 17 of the

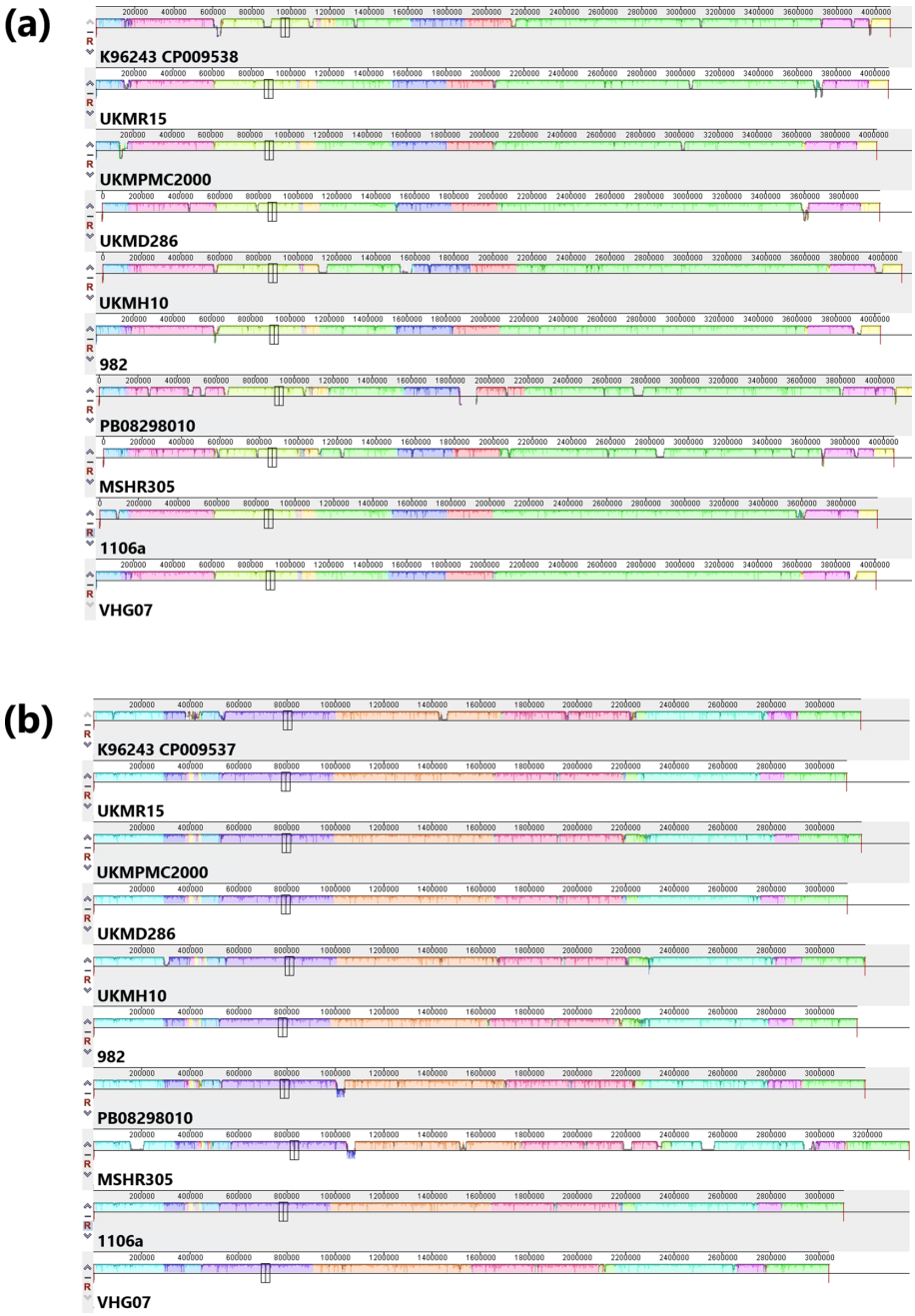

Fig. 1. Variation in chromosomal organization of the Malaysian *B. pseudomallei* isolates. (a) Chromosome 1, (b) chromosome 2. Conserved segments or LCBs that are identical across strains are visualized in the same colour, while white spaces across alignments indicate the presence of strain-specific sequences.

predicted GIs mapped to those previously identified [7, 9]. The eight predicted novel GIs contained typical GI features such as atypical DNA composition, presence of mobility genes and distinct codon usage bias, suggesting that the novel GIs predicted were not artefacts (data not shown). We noted that IslandViewer consistently tends to predict a higher number of GIs than initially reported for many different bacteria, suggesting the potential limitation of this software (Table S4). Nevertheless, we proceeded to use IslandViewer to predict the presence of GIs in the Malaysian *B. pseudomallei* genomes.

A total of 46 GIs were predicted within all Malaysian strains, with each strain containing 23 to 33 GIs (Table S5). The cumulative size of all GIs in the Malaysian strains was comparable to that of K96243, ranging from 500 to 680 kb (Table 2). All predicted GIs had a combination of GI features

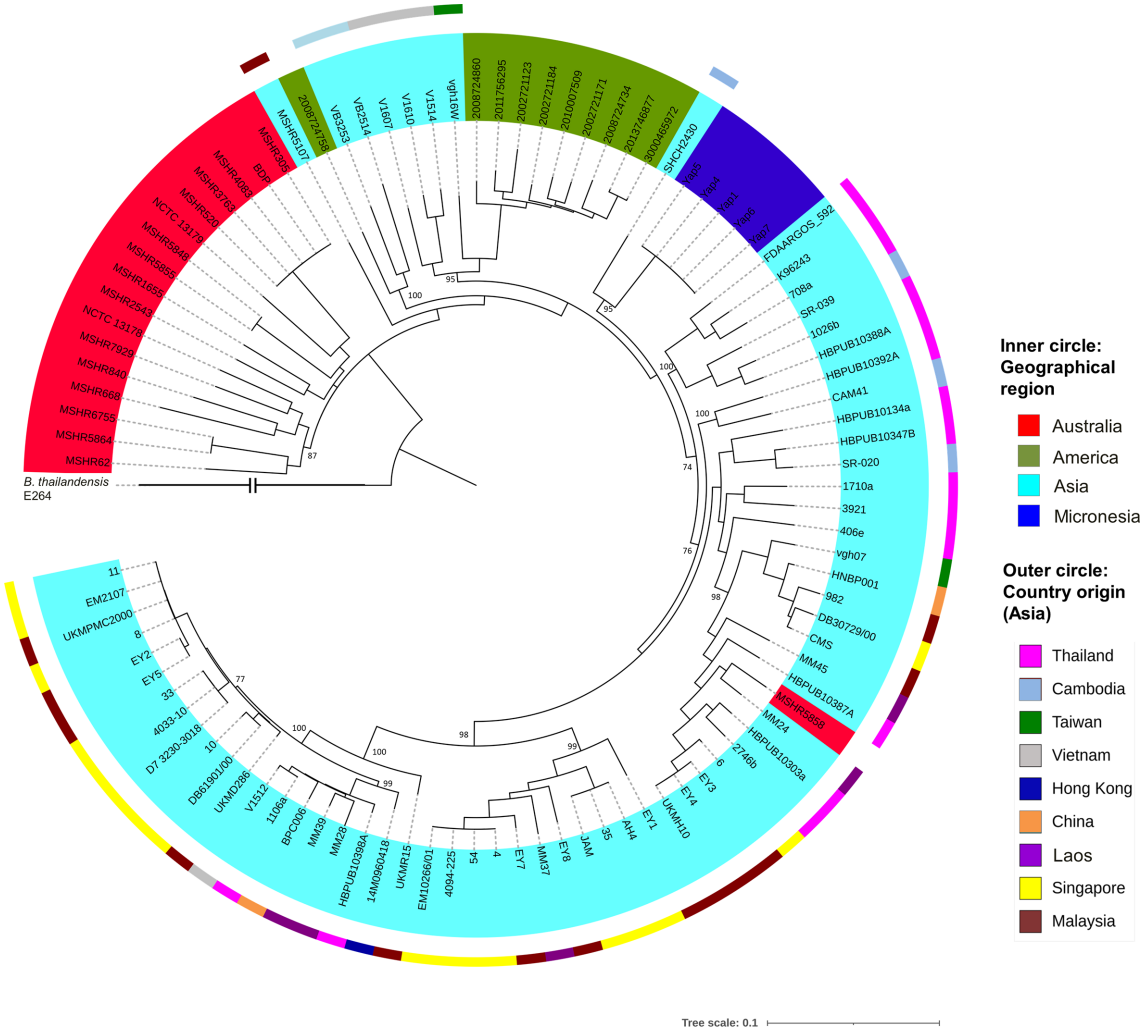

Fig. 2. Phylogenomic analysis of *B. pseudomallei* strains from Malaysia (*n*=14) and other geographical origins: Australia (red), America (light green), Micronesia (blue) and Asia (cyan). Country of origin within the Asian region is depicted in the outer circle: Thailand (magenta), Cambodia (light blue), Taiwan (green), Vietnam (grey), Hong Kong (dark blue), China (orange), Laos (purple), Singapore (yellow) and Malaysia (brown). A total of 100 *B. pseudomallei* genome sequences were used to reconstruct the phylogenomic tree using Bayesian analysis based on 230946 SNPs identified. The tree was rooted to *B. thailandensis* E264. The scale bar indicates the number of substitutions per site.

Table 2. Statistics of GIs across the Malaysian *B. pseudomallei* strains and K96243

Basic statistics	UKMD286	UKMH10	UKMPMC2000	UKMR15	K96243
No. of GIs					
Chromosome 1	15	21	16	16	12
Chromosome 2	8	12	7	7	5
Total	23	33	23	23	17
Total length (kb)	460.3	668.2	423.5	469.6	445.2
Largest GI (kb)	63.6	77.1	62.3	59.0	91.5
Mean GI (kb)	20.0	20.2	18.4	20.4	26.2

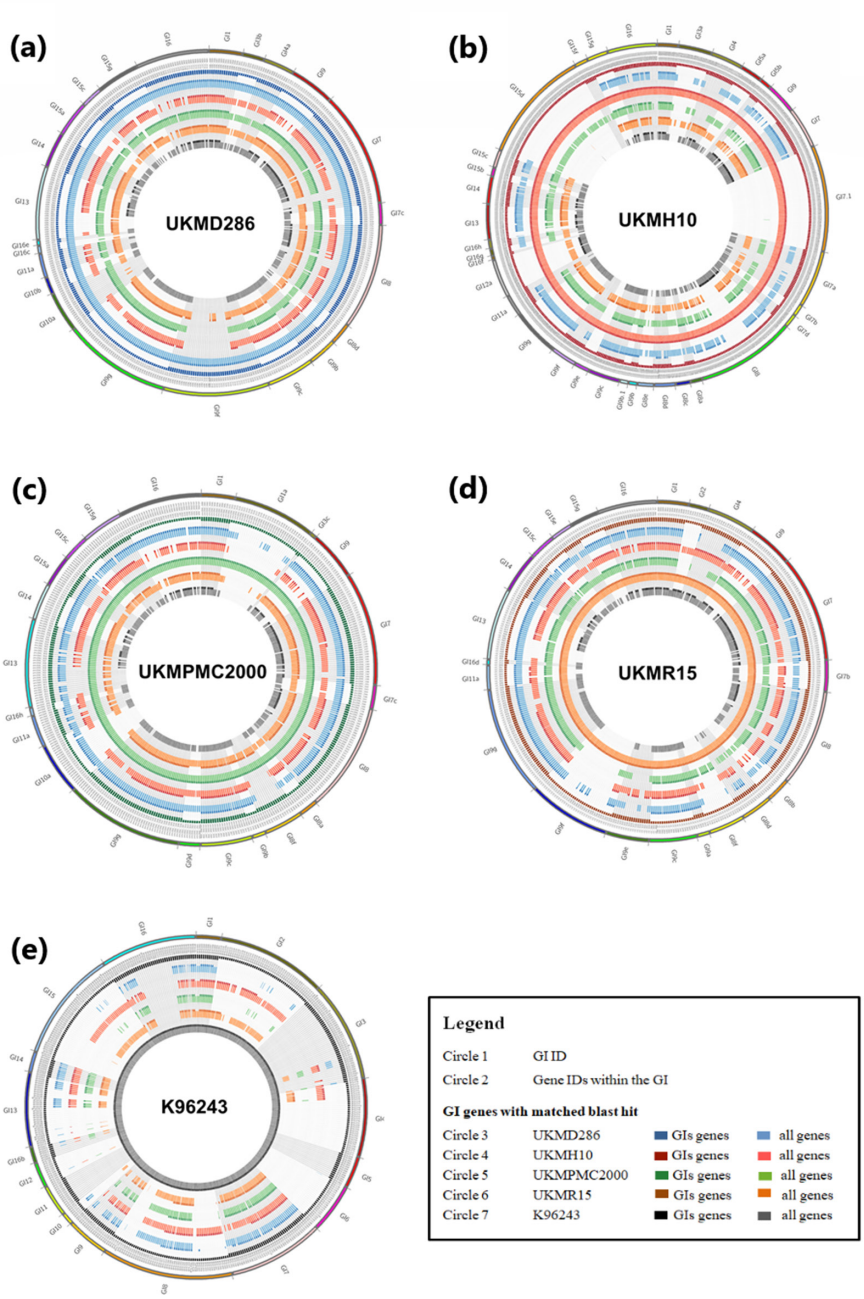

Fig. 3. Circular maps comparing GI regions across the four Malaysian *B. pseudomallei* strains, (a) UKMD286, (b) UKMH10, (c) UKMPMC2000 and (d) UKMR15, and (e) K96243.

such as a different G+C content compared with the rest of the genome, association with tRNA or transfer-messenger RNA (tmRNA) and the presence of mobility genes. Predicted GIs were labelled according to the nomenclature suggested by Tuanyok *et al.* [9] to enable GI comparisons across Malaysian strains and K96243 to identify GIs that are unique to the Malaysian strains. Overall, there is a high degree of similarity observed for GIs within K96243 and the four Malaysian strains (Fig. 3). In the Malaysian strains, GI1, GI7, GI8, GI9, GI13, GI14 and GI16 are present in all strains, while GI2 and GI4 are partially present in at least one strain. Notably GI3, GI5, GI6, GI10, GI11, GI12, GI15 and GI16b are missing in all Malaysian strains. The newly predicted GIs were found across at least one of the Malaysian strains. Some of these newly predicted GIs encode proteins with roles in metabolism or encode secondary metabolites, which may contribute to *B. pseudomallei* fitness and survival, while the rest were of unknown function (Table S5). The newly predicted GI9c was initially thought to be unique to the Malaysian strains, but further comparison with other *B. pseudomallei* genomes

demonstrated that GI9c is consistently present in all *B. pseudomallei* strains (Table S6). GI9c is AT rich and consists of genes involved in the expression of capsular polysaccharide I (CPS I), an essential virulence factor of *B. pseudomallei*. CPS I is found on the outermost layer of the bacterial cell surface and is important for mediating how the bacteria interacts with the surrounding area to ensure survival in different environments.

Interestingly, the UKMH10 genome contained a higher number of GIs, 33 GIs, compared to the other three strains with 23 predicted GIs. The total size of the 33 GIs in UKMH10 was 670 kb, while for the 23 GIs in UKMR15, UKMPMC2000 and UKMD286 it was between 420 and 460 kb. The higher number of GIs predicted within UKMH10 may be attributed to the presence of additional transposase gene sequences, a major component of insertion elements in bacteria genomes (Table S2), but this may also be a limitation of the IslandPath-DIMOB and SIGI-HMM programs. GIs were initially defined as clusters of genes within a bacterial genome that encode toxins or other pathogenicity factors acquired by HGT. Nonetheless, at this juncture, we are unable to propose that the lower virulence observed for UKMH10 is due to the absence of one or more GIs that are present in the other three strains, suggesting that the virulence capacity may be contributed by genes not within the GI regions.

Gene comparison across Malaysian *B. pseudomallei* strains

Differences in virulence levels between strains may be due to the presence or absence of genes that encode proteins that have key roles in bacterial virulence, adaption and defence. The distribution of genes that encode virulence factors across the four Malaysian strains was determined by homology searching against known *B. pseudomallei* virulence factors listed in the Virulence Factor Database (VFDB) [37] and previously reported virulence factors not found in VFDB [38]. We found no difference in the presence of genes that encode known virulence factors within the Malaysian strains with all known *B. pseudomallei* virulence-factor-encoding genes present (Table S7). This suggests that genes involved in metabolism or encoding transcription factors may contribute to the difference in virulence between UKMH10 and the other three strains. We extended the analysis to a comparison of gene content between all four strains to identify shared or unique genes. The predicted protein-encoding sequences of all assembled genomes were combined and classified into homologous clusters referred to as orthologous groups. In total, 6802 orthologues were identified across the strains (Fig. 4). A total of 5438 orthologues are shared among the Malaysian strains, while 822 orthologues are unique in the individual Malaysian *B. pseudomallei* strains.

As noted above, all four genomes contained the genes that encode known *B. pseudomallei* virulence factors and variable virulence factors (bim_{bp}/bim_{Bm} and *fhaB3*) that contribute to different melioidosis outcomes [39]. Nevertheless, our earlier study [11, 12] determined that UKMH10 is the least-virulent

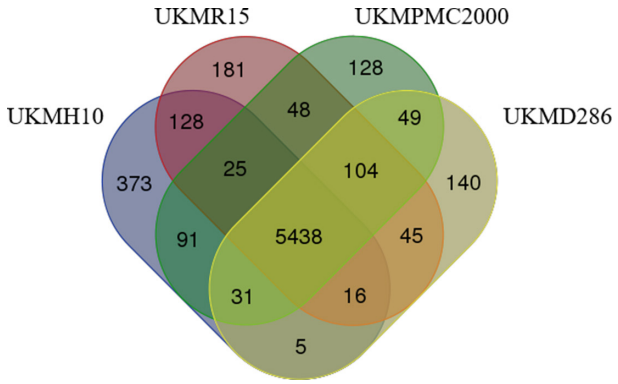

Fig. 4. Gene comparison across four Malaysian *B. pseudomallei* strains. The Venn diagram shows the intersection of orthologue groups shared between Malaysian strains. Comparison between the low-virulence strain UKMH10 with the higher-virulence group (UKMR15, UKMPMC2000 and UKMD286) shows that up to 104 genes are missing in UKMH10.

strain when compared to UKMR15, UKMPMC2000 and UKMD286. By comparing the shared and unique orthologues across the four strains, we found 104 genes absent in the UKMH10 genome, suggesting that the proteins encoded by these genes may be important for *B. pseudomallei* fitness in the host environment (Table S8). A number of these genes encode Fis family transcription regulators, LysR substrate binding proteins, as well as the RNA polymerase sigma factor. Another gene absent in UKMH10 is the *relE* gene, which is part of the type II toxin–antitoxin (TA) system that plays an important role in bacterial survival under harsh environments. TA modules usually consist of two genes organized in an operon, encoding a stable toxin and an unstable antitoxin [39]. Under normal conditions, the antitoxin inactivates the toxin by forming a TA complex. Both the antitoxin and the TA complex can act as repressors of toxin transcription. However, in conditions seen as stressful to the bacteria, the antitoxin is degraded and toxin molecules are released, inhibiting important cellular functions, causing reduced bacterial growth and leading to the formation of persister cells to ensure bacteria survival [39]. In this regard, the absence of the *B. pseudomallei* RelE protein in UKMH10 may prevent it from forming persister cells; thus, reducing its capacity to withstand harsh environments such as the infected host. The involvement of RelE in the formation of persister cells has been demonstrated previously in *B. pseudomallei* and *Escherichia coli* [40, 41].

To determine whether these 104 genes contribute to *B. pseudomallei* fitness in general, we mapped the list of genes to the genome of two other *B. pseudomallei* strains with different virulence phenotypes: NCTC13178, a highly virulent strain, and NCTC13179, a low-virulence strain [42]. All the genes were consistently present or absent in both strains (Table S8), refuting the above-proposed relationship between the gene products and *B. pseudomallei* virulence in general. Nonetheless, there remains the possibility that the association between gene loss and low virulence, as noted for UKMH10, may be

strain-specific. A number of genes absent in the UKMH10 genome do encode proteins with roles as transcription factors and defence molecules, which may be important in ensuring bacterial fitness when faced with the hostile host environment during infection.

Nucleotide sequence accession numbers

Whole-genome sequences of the four Malaysian *B. pseudomallei* strains have been submitted to the European Nucleotide Archive (ENA) under study accession number PRJEB3252. The genome sequence accession numbers are ERS3410623 (UKMR15), ERS3410624 (UKMPMC2000), ERS3410625 (UKMD286) and ERS3410626 (UKMH10).

DISCUSSION

In this study, we set out to determine whether gene level variation could account for differences in virulence between Malaysian *B. pseudomallei* strains. We assembled and compared the genomes of four Malaysian *B. pseudomallei* strains, the highly virulent strains UKMR15, UKMPMC2000 and UKMD286, with the low-virulence strain UKMH10 [11, 12]. Taking advantage of the PacBio long sequence reads, each strain was sequenced to a depth of >200× genome coverage, to enable complete genome assembly of the four isolates. Strains UKMR15, UKMPMC2000 and UKMD286 were successfully assembled into two contigs representing both chromosomes 1 and 2, while strain UKMH10 was also assembled into two large contigs representing both chromosomes albeit with a single gap of 70 kb present on chromosome 2. Gene prediction of the assembled genomes identified between 5997 and 6314 genes, which is similar to the gene content of the reference strain, *B. pseudomallei* K96243. We observed a higher number of transposons in the UKMH10 genome compared to the other strains, suggesting a larger incidence of HGT activities in UKMH10, contributing to the slightly larger genome size and higher number of GIs present. The new predicted GI, GI9c, is AT rich, 23 kb in length and consists of 20 genes with a role in CPS I expression. CPS I is a known *B. pseudomallei* virulence factor found on the outermost layer of the bacteria cell surface and is required for clinical infection, *B. pseudomallei in vivo* survival and persistence. CPS I inhibits host complement-mediated bacteria clearance to ensure bacterial survival [43]. Attenuation of virulence has been demonstrated in CPS I operon knockouts, validating the importance of this GI for *B. pseudomallei* virulence [43]. Our analysis also showed that this GI is present in pathogenic *Burkholderia* species such as *B. pseudomallei* and *Burkholderia mallei* but not in non-pathogenic *B. thailandensis*, and was previously proposed as a *B. pseudomallei* pathogenic island (PAI) [44].

The availability of these genome sequences enabled us to examine the relationship between the Malaysian strains and *B. pseudomallei* from other regions focusing on human clinical isolates. Our phylogenetic analysis agrees with previous reports, which suggested that *B. pseudomallei* originated from Australia and dispersed to Asia [8, 34, 45]. All Malaysian strains reside within the Asian clade, including MSHR5107 (isolated from a melioidosis patient in Sarawak, the Borneo region of Malaysia), which resides at the edge of the Asian clade, separated far from other Malaysian strains. This observation for MHSR5107 is similar to a previous report showing that the strain is clustered on the edge of the Asian clade, close to the Australian clade [34, 46]. The strain is of the ST881, an ST specific to Sarawak. *B. pseudomallei* strains within this ST881 group are mostly susceptible to the aminoglycoside antibiotic gentamicin, which is a rare occurrence for *B. pseudomallei* [47]. MHSR5107's linkage close to the Australia clade in the phylogenetic tree appears to suggest that the strain may be a remnant from an ancestral population that may have been introduced into the region before being dispersed to the South-East Asian region. However, more samples from the region and its neighbours are required to understand the dissemination path into this region and other locations in Asia. The Malaysian strains used in this study, UKMR15, UKMPMC2000, UKMD286 and UKMH10, all reside within the Asian clade. Nonetheless, UKMR15, UKMPMC2000 and UKMD286 are clustered in a clade that is separated from UKMH10. This observation suggests that UKMR15, UKMPMC2000 and UKMD286 are closely related compared to UKMH10, which also mirrors the differences in virulence levels between the strains [11, 12].

Earlier studies on *B. pseudomallei* genome analysis and evolution have shown that the majority of South-East Asian *B. pseudomallei* clusters can be classified as either Malay Peninsula (South Thailand, Peninsular Malaysia and Singapore) or the Mekong sub-region, referring to countries bordered by the Mekong River (Thailand, Laos, Cambodia and Vietnam) [45]. Dating of these clusters suggests that dispersion of *B. pseudomallei* within this region occurred in early 20th century. However, it is also likely that *B. pseudomallei* could have dispersed within the Asian region at a much earlier time point. For example, ST46 is a major ST group among Malaysian isolates, and can be traced back to other Asian countries including China, Vietnam, Thailand and Cambodia. This is not surprising, as archaeological findings have suggested that the South-East Asian region has seen global trading even before 1 AD [48]. Regions such as the Mekong Delta, Isthamus of Kra and Straits of Malacca served as entrepôts connecting merchants from East Asia to West Asia. During the trade exchanges, these individuals may have transported goods, animals, soil or perhaps water contaminated with *B. pseudomallei* and spread the bacteria across the region.

Most of the clinical presentations of melioidosis cases reported in Malaysia are associated with pneumonia or bacteraemia [49], There have been suggestions that different virulence genes are associated with the clinical outcomes of melioidosis. For example, $BimA_{Bp}$ is associated with pneumonia, while $BimA_{Bm}$, a variant of BimA that shares high homology with *B.mallei* BimA, is associated with neurological melioidosis [38]. We screened for the presence of $bimA_{Bp}$/$bimA_{Bm}$ and *fhaB3* in the four Malaysian strains, and all strains carry $bimA_{Bp}$ associated with pneumonia, which is highly represented in local melioidosis cases. FhaB3 has been

linked to increased severity of melioidosis [38]; however, even UKMH10 carries the *fhaB3* gene in its genome (Table S7).

The ST profile and phylogenetic analysis is indicative that that UKMR15, UKMPMC2000 and UKMD286 are closely related compared to UKMH10. This relationship also mirrors the differences in virulence levels between UKMH10 and the other three strains. UKMR15 is the most virulent, followed by UKMPMC2000 and UKMD286, while UKMH10 is the least virulent [11, 12]. The presence of more GIs and a larger total GI size in UKMH10 hints at more frequent recombination events, which is also reflected in the higher number of mobile elements present in UKMH10 (Table S2). GI variation contributes to genome plasticity, which may reflect greater adaptation properties and survival in different environments, whilst also linked to bacteria virulence or attenuation [50]. *B. pseudomallei* virulence determinants are yet to be fully resolved; however, it is assumed that variation in the number of GIs and their gene content could account for differences in virulence [51]. Even though we observed some diversity in terms of the GI content between the four strains, we could not associate these differences to the virulence of each strain. Hence, the difference in virulence may be attributable to alterations at the individual gene level or to a synergistic effect between multiple genes, including those not directly related to virulence. Changes at the gene level may be due to either the total loss of genes or indels that prevent the expression of virulence-related genes. This prompted us to confirm the presence of all known *B. pseudomallei* virulence-factor-encoding genes in the UKMH10 genome. Our search confirmed that none of the known virulence-factor-encoding genes were missing in UKMH10 (Table S7), suggesting that other genes may encode proteins that have not been revealed as virulence-factor-encoding genes. A gene-level comparison between UKMH10, UKMR15, UKMPMC2000 and UKMD286 identified 104 genes that were missing in UKMH10. Some of the missing genes encode proteins with key roles in gene regulation and the RNA polymerase sigma factor. Previous work shows that these genes might contribute to *B. pseudomallei* strain differences in virulence levels [51].

One of the absent genes is *relE*, which works in tandem with the antitoxin gene to form a TA system in *B. pseudomallei*. TA systems are commonly found in bacteria and have been implicated in bacterial pathogenicity, i.e. bacterial persistence in harsh environments [52, 53], virulence [54–56] susceptibility to antibiotics [53, 57] and cell growth [58]. *relE* encodes a toxin that targets mRNA in a ribosome-dependent manner, whereby the toxin binds to 30S ribosomes and stimulates endogenous ribonuclease activity [59]. The *Burkholderia cenocepacia* RelE toxin causes growth inhibition and contributes to biofilm production, which is associated with bacterial persistence [60]. This suggests that *B. pseudomallei* UKMH10, which lacks the *relE* gene, may not be able to persist in the dormant stage, making it vulnerable to the host defence mechanism and other forms of stress.

In conclusion, our findings demonstrate that the observed minimal (1–2%) gene loss is sufficient to render a pathogenic bacterium less virulent and that the gene loss may have arisen through multiple recombination events during HGT. The genes lost in the low-virulence strain, *B. pseudomallei* UKMH10, may play key roles linked to bacterial survival and adaptation in the eukaryotic host. Further analysis of the proteins of unknown function encoded by these genes may provide new knowledge on *B. pseudomallei* virulence and pathogenic mechanisms.

Funding information
This project was funded by the Ministry of Science, Technology and Innovation Malaysia (02-05-20-SF0006), Universiti Kebangsaan Malaysia grant number DIP-2015–022 and the Ministry of Higher Education Malaysia (FRGS/1/2016/SKK11/UKM/01/1). A-KG acknowledges the support of a MyBrain Scholarship from the Ministry of Higher Education Malaysia.

Author contributions
A.-K. G. and S. N. designed the study. S.-A. E. prepared the genomic libraries. A.-K. G., J.-S. K. and S. T. assembled, annotated and analysed the genomic sequence data. J.-S. K. performed the phylogenomic analysis. A.-K. G., S. T. and S. N. analysed the data. C.-C. H. provided the analysis tools and computing resources needed for the study. A.-K. G. and S. N. wrote the manuscript. All authors contributed to the editing and to a critical analysis of the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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